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Induction of a retinoblastoma phosphatase activity by anticancer drugs accompanies p53-independent G_1 arrest and apoptosis

(cell cycle/DNA damage/G_1 checkpoint/programmed cell death)

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ABSTRACT **DNA-damaging agents induce accumulation of the tumor suppressor and G_1 checkpoint protein p53, leading cells to either growth arrest in G_1 or apoptosis (programmed cell death). The p53-dependent G_1 arrest involves induction of p21 (also called WAF1/CIP1/SDI1), which prevents cyclin kinase-mediated phosphorylation of retinoblastoma protein (RB). Recent studies suggest a p53-independent G_1 checkpoint as well; however, little is known about its molecular mechanisms. We report that induction of a protein-serine/threonine phosphatase activity by DNA damage signals is at least one of the mechanisms responsible for p53-independent, RB-mediated G_1 arrest and consequent apoptosis. When two p53-null human leukemic cell lines (HL-60 and U-937) were treated with a variety of anticancer agents, RB became hypophosphorylated, accompanied with G_1 arrest. This was followed immediately (in less than 30 min) by apoptosis, as determined by the accumulation of pre-G_1 apoptotic cells and the internucleosomal fragmentation of DNA. Addition of calyculin A or okadaic acid (specific serine/threonine phosphatase inhibitors) or zinc chloride (apoptosis inhibitor) prevented the G_1 arrest- and apoptosis-specific RB dephosphorylation. The levels of cyclin E- and cyclin A-associated kinase activities remained high during RB dephosphorylation, supporting the involvement of a chemotherapy-induced serine/threonine phosphatase(s) rather than p21. Furthermore, the induced phosphatase activity coimmunoprecipitated with the hyperphosphorylated RB and was active in a cell-free system that reproduced the growth arrest- and apoptosis-specific RB dephosphorylation, which was inhibitable by calyculin A but not zinc. We propose that the RB phosphatase(s) might be one of the p53-independent G_1 checkpoint regulators.**

Cell numbers are regulated by a balance between proliferation, growth arrest, and apoptosis. In normal mammalian cells, proliferation is tightly controlled in the late G_1 phase of the cell cycle through a process that involves cyclins, cyclin-dependent kinases (cdks), cdk inhibitors (such as p21, also called WAF1/CIP1/SDI1), retinoblastoma protein (RB), and other proteins (1, 2). Recent evidence suggests that intracellular signals governing cell proliferation and cell cycle progression also mediate apoptosis (3). Apoptosis is an active, energy-dependent process of cellular self-destruction that involves cell shrinkage, membrane blebbing, chromatin condensation, and eventual internucleosomal DNA cleavage, or formation of a 180-bp DNA ladder. Apoptosis can be triggered by various external stimuli including DNA-damaging agents such as chemotherapeutic drugs and irradiation (3, 4).

A critical regulator of the cellular response to DNA damage is p53, a tumor suppressor and transcription factor. p53 regulates a DNA damage-triggered G_1 checkpoint (2). The levels of p53 are low in normal cells but rise rapidly after exposure to DNA-damaging agents. In some cell types, induction of p53 results in transcriptional activation of p21, which inhibits cdk-mediated RB phosphorylation, leading cells to arrest in G_1 (2, 5, 6). In other cells, p53 activates expression of Bax (a cell death inducer) and suppresses that of Bcl-2 (a cell death inhibitor), leading to cellular apoptosis (7, 8). Most recently, studies using transgenic mice suggest that loss of RB function is associated with induction of p53-dependent apoptosis (9, 10).

Recent experiments also suggest a p53-independent, DNA damage-triggered G_1 checkpoint (11). However, the molecular mechanism involved remains unknown. The p53-independent G_1 arrest may also require RB dephosphorylation, since when HL-60 and U-937 cell lines, both of which contained undetectable levels of p53 (12), were induced to differentiate, RB dephosphorylation preceded the total arrest of cell growth (13). Expression of p21 can also be induced through p53-independent pathways (14, 15), which might be one of the mechanisms responsible for RB dephosphorylation in these cells. However, it is possible that other mechanisms (such as induction of RB phosphatases) also mediate the status of RB phosphorylation. In the study presented here, we report that induction of a protein-serine/threonine phosphatase activity rather than p21 is responsible for the chemotherapy-induced RB dephosphorylation and consequent G_1 arrest and apoptosis in two p53-null human leukemic cell lines.

MATERIALS AND METHODS

Materials. Sodium vanadate was purchased from Fisher; calyculin A and okadaic acid were from Life Technologies (Grand Island, NY); and cytosine arabinoside (Ara-C), etoposide (VP-16), dimethyl sulfoxide, zinc chloride, sodium fluoride, β-glycerophosphate, and all other chemicals were from Sigma. Purified mouse monoclonal antibody to human RB protein, G3-245, was from PharMingen; mouse monoclonal culture supernatants to human RB, XZ104 and 133 (16), were a kind gift from N. Dyson and E. Harlow (Massachusetts General Hospital Cancer Center, Charlestown, MA).

Cell Culture and Drug Treatment. HL-60 human promyelocytic leukemia and U-937 human monocytic leukemia cells were grown as described (14). Ara-C (60 mM) was dissolved in phosphate-buffered saline (PBS), VP-16 (400 mM) was dissolved in dimethyl sulfoxide, and okadaic acid (0.5 mM) and calyculin A (0.1 mM) were dissolved in 10% (vol/vol) dimethyl sulfoxide. Other phosphatase inhibitors were dissolved in distilled water (1 M) and filtered before use.

Flow Cytometry and DNA Fragmentation Assays. DNA content analysis using flow cytometry was performed in the University of Pittsburgh Cancer Institute essentially according

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Abbreviations: Ara-C, cytosine arabinoside; cdk, cyclin-dependent kinase; VP-16, etoposide; RB, retinoblastoma protein.

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to published procedures (17). The DNA fragmentation assay was performed as described (18).

Western Blot, Histone H1 Kinase, and Cell-Free RB Dephosphorylation Assays. Whole-cell extracts and immunoprecipitates were prepared as described (19). The ECL Western blot assay using RB antibody G3-245 (5 μg/ml) and the H1 kinase assay using immunoprecipitates were performed as described (19). For the cell-free RB dephosphorylation assay, HL-60 cells were pretreated with Ara-C or PBS for 1 h, followed immediately by preparation of whole-cell extracts or immunoprecipitates using Ca^{2+}/Mg^{2+}-free PBS. An aliquot of a cell extract (15 μg of protein per reaction) or an immunoprecipitate (prepared from 60 μg of protein and 5 μl of monoclonal culture supernatant per reaction) was resuspended in 10 μl of the same PBS and incubated at 30°C for up to 90 min (controls kept at 4°C). After incubation, the reaction mixtures were analyzed by Western blot assay.

RESULTS

RB Dephosphorylation Accompanies G_1 Arrest and Apoptosis in HL-60 and U-937 Cells. We investigated whether RB is involved in anticancer drug-induced G_1 arrest and apoptosis in HL-60 cells. These cells were pretreated with Ara-C for 1 or 2 h, washed, and incubated in drug-free medium. At the indicated times, aliquots of cells were used for measurement of RB (by Western blot analysis), cell cycle distribution (flow cytometry), and apoptosis [assayed by induction of pre-G_1 cell population with $<G_1$ DNA content ("apoptotic" peak, by flow cytometry), 180-bp DNA ladder (agarose gel electrophoresis), and cell nonviability (trypan blue exclusion)].

A major (p120) and a minor (p110) RB band was detected in exponentially grown HL-60 cells (Oh) by using two RB monoclonal antibodies, 245 (Fig. 1*A*, lane 1) and XZ55 (data not shown). p120 was the hyperphosphorylated (p120/hyper) while p110 was the unphosphorylated (p110/hypo) form of RB, since alkaline phosphatase treatment of such a cell extract decreased the level of p120 and simultaneously increased that of p110 (data not shown). About 50% of these cells accumulated in G_1, and 4% underwent spontaneous cell death, as evident by their $<G_1$ DNA content (Fig. 1*B*) and nonviability (data not shown). During a 2-h Ara-C treatment (2h), even though no apparent RB changes were detected (Fig. 1*A*, lane 2), G_1 cells increased about 10%, accompanied with decreased population in S, G_2, and M (Fig. 1*B*; see *Discussion*). However, the drug-induced apoptosis had not been detected at this time (Fig. 1 *B* and *C*). Therefore, G_1 arrest precedes apoptosis in the drug-treated HL-60 cells.

One hour after removing Ara-C (2h+1h), p120/hyper became undetectable, and simultaneously a new, abundant band of 115 kDa was observed (p115/hypo; Fig. 1*A*, lane 3), suggesting RB dephosphorylation. At the same time, G_1 cells increased 25% (Fig. 1*B*), demonstrating a tight association between RB dephosphorylation and G_1 arrest. Apoptosis had been induced at this time, as evident by a 20% increase in apoptotic peak (Fig. 1*B*). Consistent with this, agarose gel electrophoresis assay detected DNA fragments with relatively high molecular masses (as indicated by the arrow, Fig. 1*C*, lane 3), which were later converted to a typical 180-bp ladder (lane 4). These data suggest that RB dephosphorylation, as well as apoptotic peak formation, is an early event in the process of apoptosis.

Between 1 and 3 h (2h+3h) after removing the drug, even though no further increase was detected in the levels of p115/hypo (which actually decreased slightly, Fig. 1*A*, lane 4 vs. lane 3) and G_1 arrest and apoptotic peak (data not shown), the 180-bp ladder dramatically increased (Fig. 1*C*, lane 4 vs. lane 3). More detailed kinetic experiments indicated that the DNA ladder had been increased in 30 min or less, rather than 2 h, after RB dephosphorylation (see Fig. 2). About 20 h later,

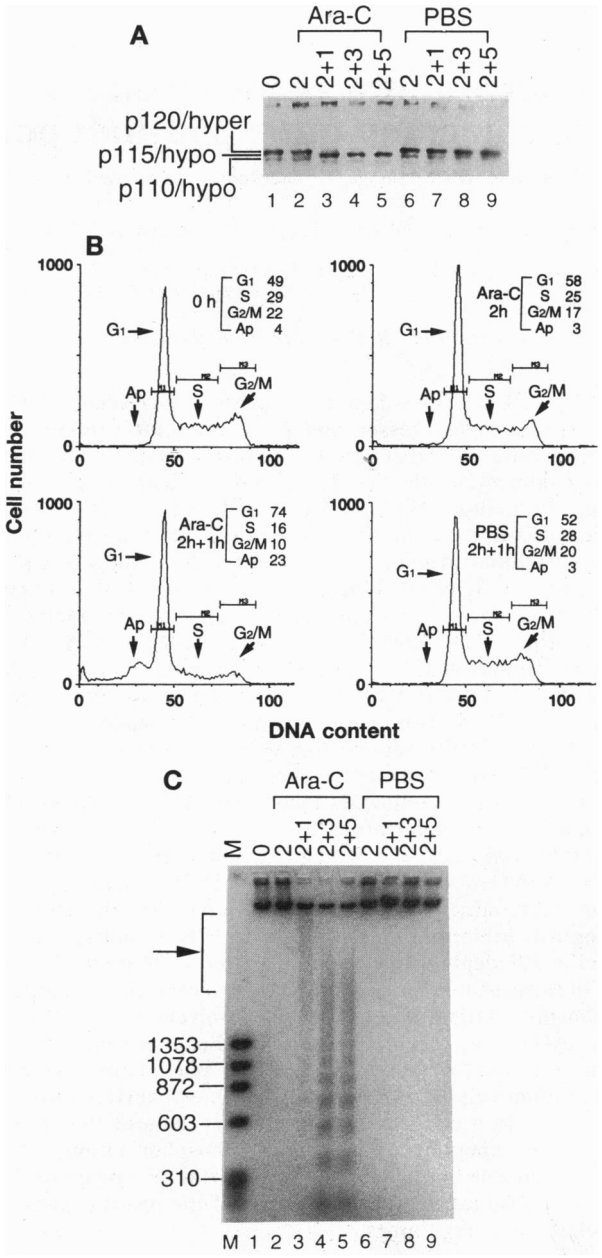

FIG. 1. Induction of RB dephosphorylation, G_1 arrest, and apoptosis by Ara-C in HL-60 cells. Exponentially grown HL-60 cells (0 h) were treated with 10 μM Ara-C or an equal percentage (0.017%) of PBS for 2 h (2h), washed, and incubated in drug-free medium for additional 1, 3, or 5 h (2+1, 2+3, 2+5, respectively). Aliquots of cells were prepared. (*A*) Western blot assay. The hyperphosphorylated (p120/hyper) and hypophosphorylated (p110/hypo) as well as the drug-induced, hypophosphorylated (p115/hypo) forms of RB are indicated. (*B*) DNA content assay. The cell cycle distribution was calculated as the percentage of cells that contain G_1, S, G_2, and M DNA judged by propidium iodide staining; the apoptotic population (Ap) was the percentage of cells with $<G_1$ DNA content, obtained from the differences between the total and cells in the cell cycle. Similar results were obtained in at least three other independent experiments. (*C*) DNA ladder assay. Lane M, DNA molecular markers (in bp). The large DNA fragments, probably the precursor DNA of the 180-bp ladder, are indicated by an arrow.

50% of cells lost their viability, as measured by incorporation of trypan blue dye (data not shown).

None of these Ara-C-induced events including RB dephosphorylation, G_1 arrest, and apoptosis were observed in cells treated with the solvent PBS (Fig. 1), demonstrating drug specificity. These events were also induced in HL-60 cells by

other anticancer drugs such as VP-16 and cisplatin (Q.P.D. and B.A., unpublished data and see Fig. 3). In addition, these events were also seen when another p53-null cell line U-937 (12) was treated with a variety of anticancer agents (see Fig. 3). Therefore, the growth arrest- and apoptosis-specific RB dephosphorylation was multidrug-induced in at least two p53-negative cell lines.

Zinc Inhibits the Chemotherapy-Induced Events. To further investigate the role of RB dephosphorylation in the process of the drug-induced growth arrest and apoptosis, we used zinc chloride, an apoptosis inhibitor (20). Addition of zinc into the Ara-C-pretreated HL-60 cells resulted in inhibition of RB dephosphorylation (Fig. 2*A*, lane 4 vs. lane 3), the apoptotic peak (Fig. 2*B*; $+Zn^{++}$ vs. $-Zn^{++}$), and the DNA ladder (Fig. 2*C*, lane 4 vs. lane 3). However, zinc did not completely overcome the drug-induced growth arrest since the percentage of the G_1 population in these cells was still 5–10% higher than that of control cells (Fig. 2*B*; see *Discussion*).

To confirm that inhibition of the apoptosis-specific DNA ladder is a biological consequence of inhibition of RB dephosphorylation, zinc was added either immediately or later in 0.5-h intervals after withdrawal of Ara-C. We found that when the Ara-C-pretreated cells had been incubated in drug-free medium for <1.5 h, addition of zinc completely inhibited both RB dephosphorylation and DNA fragmentation (Fig. 2 *A* and *C*, lanes 4–6 vs. lane 3). In contrast, after the pretreated cells had been remained in fresh medium for 1.5 h or longer, addition of zinc did not block either RB dephosphorylation or DNA ladder formation (lanes 7 and 8 vs. lane 3). Since accumulation of cells in G_1 and pre-G_1 apoptotic phases accompanied RB dephosphorylation but preceded DNA ladder formation (see Fig. 1), we conclude that these Ara-C-triggered events occur sequentially/simultaneously in <30 min in a cell.

Induction of a cdk Inhibitor May Not Be Responsible for the Induced RB Dephosphorylation. RB dephosphorylation could be due to induction of a cdk inhibitor such as p21 and consequent reduction of RB kinase activities and/or induction of an RB phosphatase activity. Since we were unable to detect p21 levels in the Ara-C-treated HL-60 cells, we measured changes in levels of cyclin E- and cyclin A-associated kinase activities, which should reflect the p21 activity (2, 5, 6). During a 1-h Ara-C treatment, RB remained hyperphosphorylated even though the levels of the cdk activities were slightly decreased (Fig. 2*A*, lane 2 vs. lane 1). After the pretreated cells were incubated in fresh medium for 3 h, RB became completely hypophosphorylated, whereas the levels of these kinase activities were not further decreased (lane 3 vs. lane 2). These data strongly suggest that the drug-induced RB dephosphorylation may not be caused by induction of a cdk inhibitor or reduction of RB kinase activities. Furthermore, while the Ara-C-pretreated cells were incubated with zinc, inhibition of RB dephosphorylation was not correlated with increases in the kinase activities (Fig. 2*A*, lanes 4–6 vs. lanes 3, 7, and 8). From these experiments, we conclude that the mechanism for the inhibitory function of zinc may not involve interfering with the cdk pathway.

Inhibitors of Protein-Serine/Threonine Phosphatases Block the G_1 Arrest- and Apoptosis-Specific RB Dephosphorylation. We then investigated possible induction of an RB phosphatase activity by anticancer drugs using different phosphatase inhibitors (Fig. 3). Calyculin A, a specific inhibitor of protein-serine/threonine phosphatases (21), effectively blocked Ara-C- or VP-16-induced RB dephosphorylation and DNA fragmentation at a very low concentration (10–50 nM) (Fig. 3 *A* and *B*, lanes 3 and 4 vs. lanes 1 and also lanes 6 and 7 vs. lane 5). Under the same conditions, the drug-induced apoptotic peak was also inhibited (data not shown). Okadaic acid (another specific serine/threonine phosphatase inhibitor; ref. 22) at a relatively higher concentration (400 nM) also prevented RB dephosphorylation, apoptotic peak, and internucleosomal DNA fragmentation (lane 10 vs. lane 9, and data not shown), even though it had no inhibitory effects at a low concentration (100 nM, lane 14). Addition of other phosphatase inhibitors, such as sodium vanadate, sodium fluoride, and β-glycerophosphate, at a very high concentration (1 mM) did not prevent these drug-induced events (lanes 11–13). An inhibitor alone had no effect under the experimental conditions (lanes 15–18), although okadaic acid at a much higher concentration (>600 nM) induced apoptosis (data not shown). These studies demonstrate that a multidrug-induced, protein-serine/threonine phosphatase activity is predominantly responsible for RB dephosphorylation, G_1 arrest, and apoptosis in these cells.

The Drug-Induced Serine/Threonine Phosphatase Activity Coimmunoprecipitates with and Dephosphorylates RB in a Cell-Free System. To investigate whether the drug-induced RB phosphatase activity is still active under cell-free conditions (*in vitro*), we prepared protein extracts from Ara-C-pretreated

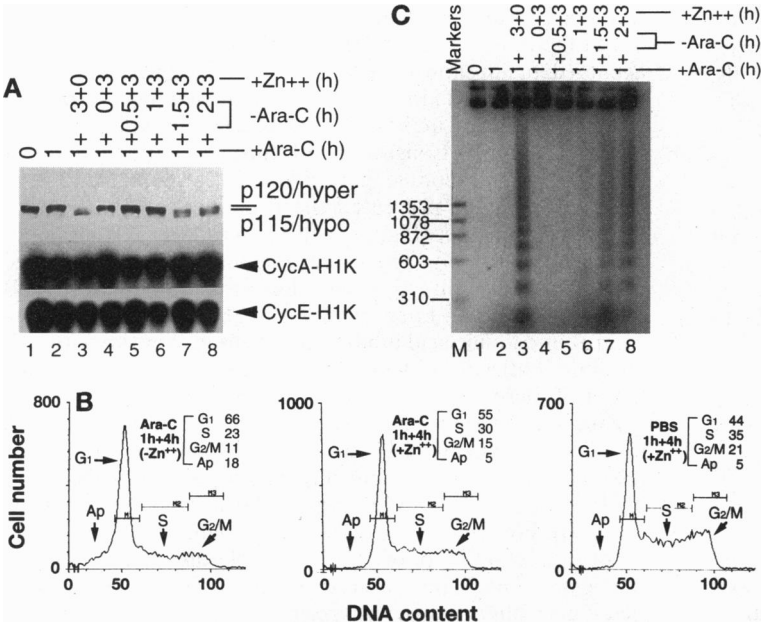

FIG. 2. Effects of zinc chloride on Ara-C-induced RB dephosphorylation, cdk activities, G_1 arrest, and apoptosis. HL-60 cells were treated with 5 μM Ara-C (+Ara-C) or PBS for 1 h and then incubated in drug-free medium for 3 or 4 hr (−Ara-C). During this period, zinc (1 mM) was added either immediately or in 0.5-h intervals, followed by a 3- or 4-h incubation ($+Zn^{++}$). Assays of RB (*A*), cyclin A- and cyclin E-associated histone H1 kinase (CycA-H1K and CycE-H1K) activities using immunoprecipitates (*A*), DNA content (*B*), and DNA ladder (*C*) were performed.

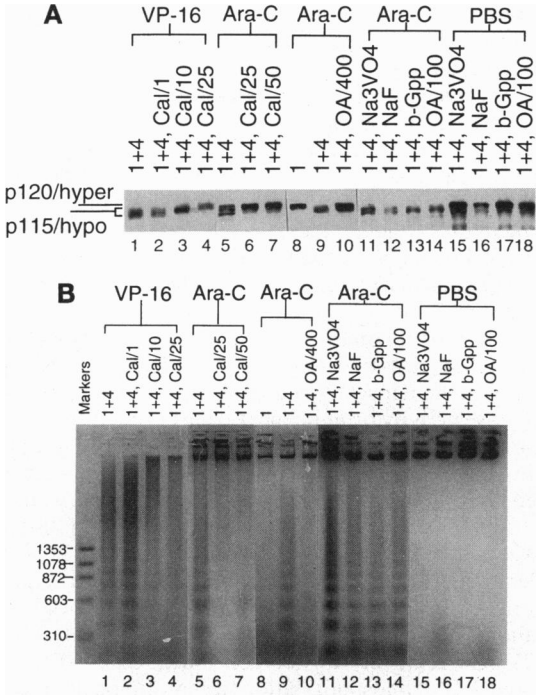

FIG. 3. Inhibition of RB dephosphorylation by protein-serine/threonine phosphatase inhibitors is tightly associated with inhibition of DNA fragmentation. U-937 (lanes 1–4) or HL-60 cells (lanes 5–18) were treated with 10 μM VP-16, 25 μM Ara-C, or an equal percentage of a solvent (such as PBS) for 1 h and then incubated in drug-free medium for 4 h (1+4), in the absence or presence of calyculin A (Cal; at a concentration of 1–50 nM), okadaic acid (OA; 100 or 400 nM), sodium vanadate (Na3VO4; 1 mM), sodium fluoride (NaF, 1 mM), or β-glycerophosphate (b-Gpp; 1 mM). Assays of RB (*A*) and DNA ladder (*B*) were performed.

HL-60 cells (*in vivo*). Incubation of such an extract at 30°C for 90 min induced RB dephosphorylation (Fig. 4*A*, lane 3), which mimicked the reaction observed in the drug-treated cells (compare to Fig. 3*A*). The RB dephosphorylation was not reproduced when the Ara-C-treated cell extract was kept at 4°C (lane 2) or when an extract, prepared from PBS-pretreated cells, was incubated at 30°C (lane 1), demonstrating a temperature-dependent, drug-specific reaction under cell-free conditions. In addition, the *in vitro*-reproduced RB dephosphorylation from cell extracts was inhibited by addition of either calyculin A or zinc (Fig. 4*A*, lanes 4–7 vs. lane 3).

To investigate whether the induced phosphatase activity associates with the hyperphosphorylated form of RB in cells, we prepared RB immunoprecipitates from extracts of Ara-C- or PBS-pretreated HL-60 cells. When these immunoprecipitates were incubated *in vitro*, a temperature-dependent, drug-specific RB dephosphorylation was reproduced (Fig. 4*B*, lane 4 vs. lanes 2 and 3). More importantly, the RB-associated phosphatase activity was inhibited only by calyculin A but not zinc (Fig. 4*B*, lanes 6 and 7 vs. lane 5 and also lanes 9 and 10 vs. lane 8). These studies confirm that a protein-serine/threonine phosphatase activity is responsible for the chemotherapy-induced RB dephosphorylation in both cell cultures (Figs. 1–3) and cell-free systems (Fig. 4). Our results also suggest that zinc might inhibit one of the upstream events that leads to activation of the serine/threonine phosphatase(s) (see *Discussion*).

DISCUSSION

We investigated the possible role of RB in anticancer drug-induced G_1 arrest and apoptosis in two p53-null leukemic cell lines, HL-60 and U-937. Our results strongly suggest that (*i*)

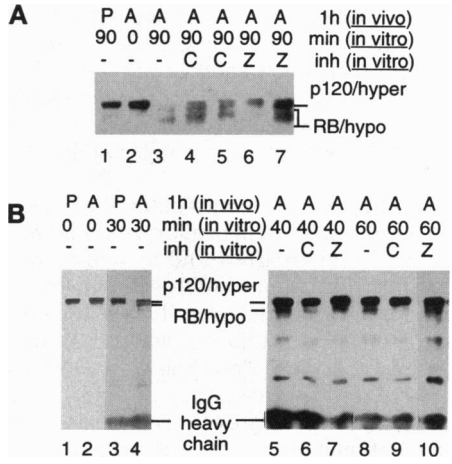

FIG. 4. RB dephosphorylation under cell-free conditions. HL-60 cells were treated with 20 μM Ara-C (A) or PBS (P) for 1 h (*in vivo*), followed by preparation of whole cell extracts (*A*) or RB immunoprecipitates (*B*). (*A*) An aliquot of the cell extracts was incubated at 30°C for 90 min (*in vitro*; 0 min represents a sample kept at 4°C) in the absence (−) or presence of calyculin A (C; 50 and 150 nM in lanes 4 and 5, respectively) or zinc (Z; 1 and 1.5 mM in lanes 6 and 7, respectively). (*B*) Immunoprecipitates were prepared by using RB monoclonal culture supernatants XZ104 (lanes 1, 2, and 5–10) or 133 (lanes 3 and 4). An aliquot of the immunoprecipitates was incubated at 30°C for the indicated times (*in vitro*), in the absence (−) or presence of calyculin A (C; 150 nM) or zinc (Z; 1 mM). After incubation, the reaction mixtures were analyzed by Western blot assay. The hyperphosphorylated and hypophosphorylated forms of RB as well as IgG heavy chain are indicated.

RB dephosphorylation is required for multidrug-induced G_1 arrest and apoptosis in these cells (Figs. 1–3), (*ii*) RB dephosphorylation is caused by induction of a protein-serine/threonine phosphatase activity, rather than induction of p21 or reduction of cdks (Figs. 2–4), and (*iii*) the induced serine/threonine phosphatase activity associates with the hyperphosphorylated RB in cells and is able to reproduce the growth arrest- and apoptosis-specific RB dephosphorylation under cell-free conditions (Fig. 4).

A Serine/Threonine Phosphatase Activity Is Responsible for the Chemotherapy-Induced RB Dephosphorylation in Two p53-Null Cell Lines. DNA damage-triggered, p53-dependent induction of p21 leads to RB dephosphorylation and consequent G_1 arrest (2, 5, 6). Even though p21 can also be induced through p53-independent pathways (14, 15), we could not detect the levels of p21 in HL-60 cells treated with anticancer agents (data not shown). However, we found that cyclin E- and cyclin A-associated kinase activities remained high during RB dephosphorylation in these cells (Fig. 2*A*), suggesting that p21 is not responsible. Consistent with this, a specific inhibitor of protein-serine/threonine phosphatases, calyculin A (21) or okadaic acid (22), prevented the drug-induced RB dephosphorylation in cells (Fig. 3), cell extracts, and RB immunoprecipitates (Fig. 4). Therefore, the serine/threonine phosphatase activity is directly responsible for the drug-induced RB dephosphorylation. Even though it has been shown that calyculin A or okadaic acid inhibits apoptosis in some systems (23, 24), our studies have demonstrated that the major apoptotic target of these inhibitors is the RB phosphatase(s).

Zinc inhibited RB dephosphorylation in cells (Fig. 2) and in protein extracts (Fig. 4*A*) but not in RB immunoprecipitates (Fig. 4*B*). Even though probably only a portion of the phosphatase activity is associated with RB in these experiments (compare Fig. 4*B* to 4*A*), these studies demonstrate that zinc is not a direct inhibitor of the RB phosphatase activity. One of the possible mechanisms for the suppressive activity of zinc is that it may inhibit a protein-tyrosine phosphatase activity (25),

which might control activation of the serine/threonine phosphatase activity. Consistent with this hypothesis, phosphorylation of RB has been found only in serine and threonine but not tyrosine residues (26). Regulation of RB phosphorylation by protein-serine/threonine phosphatases has been found to play a role in cell cycle progression (27, 28). The current studies have provided evidence for involvement in apoptosis of the serine/threonine phosphatase-mediated RB dephosphorylation.

RB Dephosphorylation Is Required for G_1 Arrest in p53-Negative Cells. We have found that accumulation of a specific hypophosphorylated form of RB, p115/hypo, accompanied G_1 arrest in the p53-null cell lines HL-60 and U-937 (Figs. 1 and 2). Inhibition of RB dephosphorylation is associated with inhibition of G_1 arrest (Fig. 2), indicating that production of p115/hypo is at least partially responsible for p53-independent G_1 arrest. Production of p115/hypo was not always correlated well with accumulation of cells in G_1. For example, when cells were treated with Ara-C for 1 or 2 h, or after the Ara-C-pretreated cells were incubated with zinc, RB was present mainly in the hyperphosphorylated form (p120/hyper), but the percentage of G_1 population in these cells was 5–10% higher than that of control cells (Figs. 1 and 2). This inconsistency might be due to the fact that Western blot assay is less sensitive for detecting 5–10% of RB changes than flow cytometry for the G_1 peak. Alternatively, there might be an additional molecular mechanism, parallel to RB pathway, involved in controlling G_1 arrest in these p53-negative cells.

Apoptosis Occurs Immediately After G_1 Arrest in p53-Negative Cells. Induction of p53 by DNA damage leads cells to either G_1 arrest or apoptosis through differentially regulating p21 or Bax/Bcl-2 (see the Introduction). In contrast, the p53-independent G_1 arrest is tightly associated with apoptosis (11). We have found that anticancer agent-induced G_1 arrest in HL-60 and U-927 cells started just prior to apoptosis (Fig. 1), supporting the hypothesis that G_1 arrest may be prerequisite for apoptosis in at least a portion of these p53-negative cells (11). In addition, inhibition of RB dephosphorylation and G_1 arrest is tightly associated with inhibition of the apoptotic peak and of the DNA ladder (Figs. 2 and 3), suggesting that a cascade of these events occurs (within <30 min) during the initiation of G_1 arrest and apoptosis.

Our data strongly suggest that anticancer drugs induce a serine/threonine phosphatase activity, which is predominantly responsible for p53-independent RB dephosphorylation, G_1 arrest, and apoptosis in HL-60 and U-937 cells. Similar to the role of p21 in controlling the p53-dependent G_1 arrest (2, 5, 6), the RB phosphatase(s) alone, or in cooperation with other regulators (such as p21), might mediate the p53-independent G_1 arrest process. Since the p53-independent apoptosis is tightly associated with G_1 arrest (ref. 11; Figs. 1 and 2), it is possible that the RB phosphatase(s) as well as RB itself might also be involved in regulating the programmed cell death in these cells. Therefore, understanding the nature of the RB phosphatase(s) is very important for understanding the molecular basis of the p53-independent checkpoint.

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